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(54) Title: TRANSGENIC PRODUCTION OF ANTIBODIES IN MILK

(57) Abstract

A method for the production of monoclonal antibodies in mammal's milk, through the creation of transgenic animals that selectively express foreign antibody genes in mammary epithelial cells.

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TRANSGENIC PRODUCTION OF ANTIBODIES IN MILK

Field of the Invention

This invention pertains to a method for the production of monoclonal antibodies in mammal's milk, specifically through the creation of transgenic animals that selectively express foreign antibody genes in mammary epithelial cells.

Background of the Invention

Immunoglobulins are heteropolymeric proteins that are normally synthesized, modified, assembled, and secreted from circulating B lymphocytes. Using recombinant DNA technology, it is possible to program cells other than B-lymphocytes to express immunoglobulin genes. The difficulties encountered in this effort stem from several factors: 1) Both heavy and light chains of immunoglobulins must be co-expressed at appropriate levels; 2) Nascent immunoglobulin polypeptides undergo a variety of co- and post-translational modifications that may not occur with sufficient fidelity or efficiency in heterologous cells; 3) Immunoglobulins require accessory chaperone proteins for their assembly; 4) The synthetic and secretory capacity of the cell may be inadequate to secrete large amounts of heterologous proteins; and 5) The secreted immunoglobulins may be unstable in the extracellular milieu of a foreign cell.

Because immunoglobulins have many therapeutic, diagnostic and industrial applications, there is a need in the art for expression systems in which these proteins can be reproducibly manufactured at a high level, in a functional configuration, and in a form that allows them to be easily harvested and purified. The development of transgenic animal

technology has raised the possibility of using large animals as genetically programmed protein factories. P.C.T. application WO 90/04036 (published 4/19/90) discloses the use of transgenic technology for immunoglobulin expression. WO 92/03918 (3/19/92) and WO 93/12227 (6/24/93) teach the introduction of unrearranged immunoglobulin genes into the germline of transgenic animals. The use of intact immunoglobulin genes (including their respective promoter regions) will result in their expression in lymphocytes and secretion into the bloodstream of the host animal; this necessitates a strategy for suppressing the expression of the host's endogenous immunoglobulins, and raises the problem of purifying the immunoglobulins from serum, which contains many other proteins, including proteolytic enzymes. Furthermore, if the transgenic approach is chosen, heavy and light chain genes must both be incorporated into the host genome, in a manner that enables their comcomittant expression.

Another option in creating transgenic animals is to link the gene of interest to a heterologous transcriptional promoter that only functions in a defined cell type within the host. In this manner, tissue-specific expression of the transgene may be programmed. U.S. Patent No. 4,873,316 (issued October 10, 1989) discloses the production of recombinant tissue plasminogen activator (TPA) in the milk of transgenic mice in which the TPA gene is linked to the promoter of the milk protein casein. Other proteins that have been expressed in a similar fashion include cystic fibrosis transmembrane conductance regulator (DiTullio et al., *Bio/Technology* 10:74, 1992), urokinase (Meade et al., *Bio/Technology* 8: 443, 1990), interleukin-2 (Buhler et al., *Bio/Technology* 8:140, 1990), and antihemophilic factor IX (Clark et al., *Bio/Technology* 7:487, 1989). Notably, these proteins are all simple single-chain polypeptides that do not require multimerization or assembly prior to secretion.

It has now been found that when a transgenic mammal is created carrying paired immunoglobulin light and heavy chain

genes under the control of the casein promoter, such an animal produces large amounts of assembled immunoglobulins which are secreted in its milk. Using the DNA constructs of the present invention, a surprisingly high efficiency of co-integration of heavy and light chain genes is observed. Using the metod and constructs of the present invention, it is possible for the first time to program a mammary epithelial cell to produce and assemble complex tetrameric glycoproteins and secrete them in high quantities.

Accordingly, it is an object of the present invention to provide methods for the large-scale production of immunoglobulins in the milk of transgenic mammals.

Another object of the invention is to provide methods for the design of synthetic immunoglobulins that can be produced in large quantities in milk.

Yet another object of the invention is to provide methods for administering therapeutically beneficial antibodies to suckling young, by creating female mammals that excrete such antibodies into their milk.

A further object of the invention is a transgenic non-human mammal having germ and somatic cells with recombinant DNA sequences encoding immunoglobulin light and heavy chains, where said sequences are operatively linked at their 5' termini to a mammary specific promoter and at their 3' end to a sequence comprising a polyadenylation site.

A further object of the invention is a casein promoter cassette comprising in the 5' to 3' direction:

a) 5' promoter sequences from the beta casein gene,
b) an XhoI restriction site, and
c) 3' untranslated sequences from the goat beta casein gene.

These and other objects of the present invention will be apparent to those of ordinary skill in the art in light of the present specification, drawings, and claims.

Brief Description of the Drawings

Figure 1 is a schematic representation of the Bc62 plasmid, which contains a 13.9 kb Sal I fragment that comprises cDNA encoding immunoglobulin light chain, flanked on its 5' and 3' termini by goat beta casein sequences.

Figure 2 is a schematic representation of the Bc61 plasmid, which contains a 14.6 kb Sal I fragment that comprises cDNA encoding immunoglobulin heavy chain, flanked on its 5' and 3' termini by goat beta casein sequences.

Figure 3 depicts the immunoblot detection of human immunoglobulin heavy chain in the milk of transgenic mice that were created using the beta casein promoter-linked immunoglobulin genes shown in Figures 1 and 2.

Figure 4 depicts the immunoblot detection of human immunoglobulin light chain in the milk of transgenic mice that were created using the beta casein promoter-linked immunoglobulin genes shown in Figures 1 and 2.

Summary of the Invention

In one aspect, this invention comprises a method for obtaining heterologous immunoglobulins from the milk of transgenic mammals. Another aspect of the prevent invention comprises the method for creating transgenic mammals by introducing into their germline immunoglobulin cDNA linked to a milk-specific promoter.

In another aspect, the present invention comprises transgenic mammals having germ cells and somatic cells having recombinant DNA sequences comprising immunoglobulin cDNA linked to a milk-specific promoter.

In still another aspect, the present invention comprises an isolated DNA comprising an expression cassette having 5' and 3' non-coding sequences derived from the goat beta casein gene linked via a unique restriction site that serves as a convenient cloning site for immunoglobulin coding sequences.

Detailed Description of the Invention

All patent applications, patents and literature cited in this specification are hereby incorporated by reference in

their entirety. In the case of inconsistencies, the present disclosure will prevail.

The present invention pertains to a method for the production of monoclonal antibodies that are excreted into the milk of transgenic animals and the method for production of such animals. This is achieved by engineering DNA constructs in which DNA segments encoding specific paired immunoglobulin heavy and light chains are cloned downstream of a promoter sequence that is preferentially expressed in mammary epithelial cells. The recombinant DNAs containing the promoter-linked heavy and light chain genes are then coinjected into preimplantation embryos. The progeny are screened for the presence of both transgenes. Representative females from these lines are then milked, and the milk is analyzed for the presence of the monoclonal antibody. In order for the antibody to be present, both heavy and light chain genes must be expressed concurrently in the same cell. The antibodies may be purified from the milk, or the milk itself, comprising the immunoglobulins, may be used to deliver the antibodies to a recipient. This is discussed below.

The immunoglobulin genes useful in the present invention may be obtained from natural sources e.g. individual B cell clones or hybridomas derived therefrom. Alternately, they may comprise synthetic single-chain antibodies in which the light and heavy variable regions are expressed as part of a single polypeptide. Furthermore, recombinant antibody genes may be used that have been predictively altered by nucleotide substitutions that do or do not change the amino acid sequence, by addition or deletion of sequences, or by creation of hybrid genes in which different regions of the polypeptide are derived from different sources. Antibody genes by their nature are extremely diverse, and thus naturally tolerate a great deal of variation. It will be appreciated by those skilled in the art that the only limitation for producing an antibody by the method of the present invention is that it must assemble into a functional configuration and be secreted in a stable form into the milk.

The transcriptional promoters useful in practicing the present invention are those promoters that are preferentially activated in mammary epithelial cells, including promoters that control the genes encoding milk proteins such as caseins, beta lactoglobulin (Clark et al., (1989) *Bio/Technology* 7: 487-492), whey acid protein (Gordon et al., (1987) *Bio/Technology* 5: 1183-1187), and lactalbumin (Soulier et al., (1992) *FEBS Letts.* 297: 13). Casein promoters may be derived from the alpha, beta, or kappa casein genes of any mammalian species; a preferred promoter is derived from the goat beta casein gene (DiTullio, (1992) *Bio/Technology* 10:74-77).

For use in the present invention, a unique XhoI restriction site is introduced at the 3' terminus of the promoter sequence to allow the routine insertion of immunoglobulin coding sequences. Preferably, the inserted immunoglobulin gene is flanked on its 3' side by cognate genomic sequences from a mammary-specific gene, to provide a polyadenylation site and transcript-stabilizing sequences. Transcription of the construct in vivo results in the production of a stable mRNA containing casein-derived 5' untranslated sequences upstream of the translational initiator codon of the immunoglobulin gene and 3' untranslated sequences downstream of the translational termination codon of the immunoglobulin gene. Finally, the entire cassette (i.e. promoter-immunoglobulin-3' region) is flanked by restriction sites that enable the promoter-cDNA cassette to be easily excised as a single fragment. This facilitates the removal of unwanted prokaryotic vector-derived DNA sequences prior to injection into fertilized eggs.

The promoter-linked immunoglobulin heavy and light chain DNAs are then introduced into the germ line of a mammal e.g. cow, sheep, goat, mouse, oxen, camel or pig. Mammals are defined herein as all animals, excluding humans, that have mammary glands and produce milk. Mammalian species that produce milk in large amounts over long periods of time are preferred. Typically, the DNA is injected into the pronuclei of fertilized

eggs, which are then implanted into the uterus of a recipient female and allowed to gestate. After birth, the putative transgenic animals are tested for the presence of the introduced DNA. This is easily achieved by Southern blot hybridization of DNA extracted from blood cells or other available tissue, using as a probe a segment of the injected gene that shows no cross hybridization with the DNA of the recipient species. Progeny that show evidence of at least one copy of both heavy and light-chain immunoglobulin genes are selected for further analysis.

Transgenic females may be tested for immunoglobulin secretion into milk, using any of the immunological techniques that are standard in the art (e.g. Western blot, radioimmunoassay, ELISA). The anti-immunoglobulin antibodies used in this analysis may be polyclonal or monoclonal antibodies that detect isolated heavy or light chains or others that react only with fully assembled (H_2L_2) immunoglobulins.

The recombinant immunoglobulins are also characterized with respect to their functionality, i.e. binding specificity and affinity for a particular antigen. This is achieved using immunological methods that are standard in the art, such as Scatchard analysis, binding to immobilized antigen, etc. The stability characteristics of an immunoglobulin in the milk of a given species are also assayed, by applying the above-described detection methods to milk that has been incubated for increasing times after recovery from the animal.

The immunoglobulins produced by the methods of the present invention may be purified from milk, using adsorption to immobilized Protein G, column chromatography, and other methods known to those of ordinary skill in the art of antibody purification.

The level of production of recombinant immunoglobulins in an individual transgenic mammal is primarily determined by the site and manner of integration of the transgene after injection into the fertilized egg. Thus, transgenic progeny derived from different injected eggs may vary with respect to this parameter.

The amount of recombinant immunoglobulin in milk is therefore monitored in representative progeny, and the highest-producing females are preferred.

Those skilled in the art will recognize that the methods of the present invention can be used to optimize the production of natural and synthetic immunoglobulins. The steps of creating a transgenic animal, testing for the presence of both heavy and light-chain genes, assaying the secretion of immunoglobulin into the milk of female progeny, and, finally, assessing the quality of the resulting antibodies, can be repeated sequentially, without undue experimentation, to establish preferred constructs for different applications.

According to the present invention, the nature of the recombinant immunoglobulins and their specific mode of use can vary. In one embodiment, the present invention encompasses high-level expression of antibodies that are harvested and purified from milk and used in purified form. High-level expression is defined herein as the production of about 1 mg/ml of protein. In another embodiment, antibodies are engineered that provide protection to humans against infectious diseases; therapeutic administration is then achieved by drinking the milk. In a still further embodiment, lactating animals are engineered to produce antibodies specifically beneficial to their offspring, which acquire them through suckling. In a still further embodiment, animals produce an antibody that protects the lactating mammal itself against breast pathogens e.g. bacteria that produce mastitis.

The unexpectedly high-volume expression of immunoglobulins using the method and constructs of the present invention also allows the use of such immunoglobulins in pharmaceutical and chemical settings. By way of non-limiting example the method of the present invention can be used to produce high levels of tetrameric antibodies directed against various pathogens (e.g. *E. coli*, *Salmonella*, hepatitis B virus), biologically active peptides (e.g. erythropoietin, tissue

plasminogen activator, gamma interferon) and for use in chemical reactions directed against various enzymes. Monoclonal antibodies that bind to the transition state of a chemical reaction can be used in industrial-scale production. Furthermore, monoclonal antibodies are often immobilized on columns for use in the purification of biopharmaceuticals; in such cases, production of the antibodies represents a significant fraction of the cost of purification. The methods of the present invention facilitate the production of high-volume, low cost antibody stocks for use in these types of applications.

The present invention is further described in the following working examples, which are intended to illustrate the invention without limiting its scope.

Example 1: Construction of a Milk-Specific Promoter Cassette

The present invention encompasses a recipient vector into which many different immunoglobulin genes can be interchangeably inserted. The vector contains 5' milk-specific promoter sequences and 3' untranslated genomic sequences that flank an XhoI cloning site. This cloning is unique because it is the only one present in the vector. Preferably, the entire expression cassette should be flanked by restriction sites that allow the easy excision of the promoter-linked immunoglobulin gene.

In this Example, the promoter and 3' genomic sequences were derived from the goat beta casein gene. The gene was cloned and characterized as described by Roberts et al., 1992, *Gene* 121:255-262, which is hereby incorporated by reference.

The expression cassette, prior to insertion of immunoglobulin genes, consists of 6.2 kb upstream of the translational start of the beta casein coding sequence and 7.1 kb of genomic sequence downstream of the translational stop of the beta casein gene. The TaqI site just upstream of the translational start codon was changed to an XhoI site. This unique XhoI cloning site is at the junction of the upstream and downstream sequences. It is this XhoI site, included in the

sequence CGCGGATCCTCGAGGACC, into which recombinant immunoglobulin genes are inserted. (D. Tullio, (1992) *Bio/Technology* **10**:74-77)

The 3' beta casein region begins at the PpuMI site found in Exon 7 and continues for 7.1 kb downstream. Included in this sequence are the remaining 18 bp of Exon 7, and all of Exon 8 and Exon 9. These encode the 3' untranslated regions of the goat beta casein gene, and terminate with the sequence: TAAGGTCCACAGACCGAGACCCACTCACTAGGCAACTGGTCCGTCCAGCTGTTAAGTGA.

To engineer restriction sites flanking the casein cassette, the goat beta casein control sequences were first cloned into the SuperCosl vector (#251301, Stratagene, La JollA, CA) with flanking NotI and SaII sites. This plasmid was then modified by changing the NotI site to a SaII site. This created a 13.3 kb SalI fragment containing the beta casein expression cassette within the gbc163 vector.

Example 2: **Construction of Promoter-linked Monclonal Antibody Genes**

In this Example, the genes encoding a human monoclonal antibody directed against a colon cancer cell-surface marker were linked to the casein promoter. cDNAs encoding the light and heavy chains of this antibody were cloned from an antibody-secreting hybridoma cell line into a pUC19-derived vector. The light and heavy chain cDNAs were present on HindIII/EcoRI fragments of 702 bp and 1416 bp, respectively.

To adapt the genes for insertion into the casein promoter cassette, XhoI restriction sites were engineered at both ends of each DNA segment as detailed below. In the same step, the region upstream of the immunoglobulin translation initiation codon was modified so that it contained sequences similar to those in the analogous region of the beta casein gene.

Light chain gene: The pUC19 plasmid containing the light chain cDNA insert was digested with HindIII, blunt-ended by treatment with the Klenow fragment of DNA Polymerase I, and

ligated to an oligonucleotide containing an XhoI recognition sequence (#1030, New England Biolabs, Beverly, MA).

The region immediately upstream of the initiating ATG was then mutagenized using an oligonucleotide with the following sequence: 5' AGT GAA TTC ATG CTC GAG AGC CAT GGC CTG GATC 3'. Digestion of the final plasmid with XhoI produced the modified light chain cDNA that was flanked by Xhol cohesive ends.

The light chain cDNA was then inserted into the unique XhoI cloning site of the gbc163 expression vector described in Example 1, yielding plasmid Bc62 (Figure 1).

Heavy chain gene: The pUC19 plasmid containing the heavy chain cDNA was mutagenized using an oligonucleotide with the following sequence: 5' AGT GAA TTC ATG CTC GAG AGC CAT GAA GCA CCTG 3'. The resulting plasmid contains an XhoI site upstream of the heavy chain translation initiation codon.

The downstream HindIII site was converted to an Xhol site using a synthetic adapter with the sequence 5' AGC TCC TCG AGG CC 3'. Digestion of the modified plasmid with XhoI produced the the 1.4 kb modified heavy chain cDNA flanked by XhoI cohesive ends. This fragment was then inserted into the unique XhoI cloning site of gbc163 to yield Bc61 (Figure 2).

Prior to injection, promoter-linked light and heavy chain genes were isolated from Bc61 and Bc62, respectively, by digestion with SalI. The fragments were then purified by gel electrophoresis followed by CsCl equilibrium gradient centrifugation. The DNA was dialyzed extensively against distilled water prior to quantitation.

Example 3: Production of Transgenic Mice

The casein promoter-linked DNA fragments encoding the immunoglobulin heavy and light chains, obtained as described in Example 2, were injected into fertilized mouse eggs using procedures that are standard in the art, as described in Hogan, B., Constantini, F., and Lacey, E., *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratories, 1986). The resulting progeny were then analyzed for the

presence of both antibody gene sequences. DNA was extracted from tail biopsy material and probed using Southern blot analysis. The probes used in the hybridization were the original cDNAs encoding the heavy and light chains. As seen in Table 1, most of the first generation transgenic progeny had incorporated both transgenes.

Table 1

Summary of Bc61 - Bc62 Mice

Founder	Sex	Bc61	Bc62	Expression
1-2	M	Pos.	Pos.	
1-3	M	Pos.	Pos.	light chain only
1-9	M	Pos.	Pos.	
1-15	F	Neg.	Pos.	Low level lambda chain
1-16	F	Pos.	Neg.	
1-19	F	Pos.	Pos.	N.D.
1-23	F	Pos.	Pos.	1-3 mg/ml
1-24	F	Pos.	Pos.	low level
1-25	M	Pos.	Neg.	
1-39	M	Pos.	Pos.	
1-13	F	Pos.	Pos.	N.D.
1-56	F	Pos.	Pos.	N.D.
1-64	M	Pos.	Pos.	
2-76	F	Pos.	Pos.	1-3 mg/ml
2-82	F	Pos.	Pos.	1-3 mg/ml
1-72	M	Pos.	Pos.	
2-92	F	Pos.	Pos.	0.2 - 0.5 mg/ml
2-95	F	Pos.	Pos.	0.2 - 0.5 mg/ml

N.D. = not detected

Example 4: **Analysis of Recombinant Immunoglobulins in Milk**

Samples of milk from the transgenic mice obtained as described in Example 3 were analyzed for the presence of the heterologous immunoglobulin by Western blot. The heavy chain of the antibody was detected using a horseradish peroxide-linked polyclonal antibody directed against human gamma heavy chain (Antibody #62-8420, Zymed, South San Francisco, CA.) as shown in Figure 3. The light chain was detected using antibodies to the human lambda light chain, (Antibody #05-4120, Zymed, South San Francisco, CA) shown in Figure 4. In these Figures, it can be seen that immunoreactive heavy and light chains can be detected in the milk of several animals, but not in the negative control animal CD-1. Human immunolglobulin can be detected in milk from founder 1-23 and from the progeny of the 1-76 and 1-72 founders. These animals are the second-generation females, 2-76, 2-82, 2-92, and 2-95. The levels of expression range between 0.2 mg/ml to over 1 mg/ml (Table 1).

What is claimed is:

1. A method for obtaining heterologous immunoglobulin from the milk of a transgenic mammal comprising the steps of:

a. introducing into the germline of said mammal DNA comprising the protein-coding sequences of said immunoglobulin, said DNA operatively linked at its 5' terminus to a promoter sequence that supports the preferential expression of said genes in mammary gland epithelial cells, and said DNA operatively linked at its 3' terminus to a sequence containing a polyadenylation site, and

b. obtaining milk from said mammal.

2. The method of claim 1 wherein said mammal is selected from the group consisting of mice, cows, sheep, goats, oxen, camels, and pigs.

3. The method of claim 1 wherein said promoter is selected from the group consisting of the casein promoter, the beta lactoglobulin promoter, the whey acid protein promoter, and the lactalbumin promoter.

4. The method of claim 1 wherein said immunoglobulin comprises heavy and light chains.

5. The method of claim 1 wherein said immunoglobulin comprises a single polypeptide chain.

6. The method of claim 1 wherein said immunoglobulin is of human origin.

7. The method of claim 1 wherein said immunoglobulin is purified from the milk of said mammal.

8. A transgenic non-human mammal all of whose germ cells and somatic cells contain recombinant DNA sequences

encoding immunoglobulin heavy and light chains, wherein said sequences are operatively linked at their 5' termini to a promoter sequence that supports the preferential expression of said genes in mammary gland epithelial cells, and operatively linked at their 3' termini to a sequence containing a polyadenylation site.

9. The transgenic mammal of claim 8 wherein said mammal is selected from the group consisting of mice, cows, sheep, goats, oxen, camels, and pigs.

10. The transgenic mammal of claim 8 wherein said promoter is selected from the group consisting of the casein promoter, the beta lactoglobulin promoter, the whey acid protein promoter, and the lactalbumin promoter.

11. The transgenic mammal of claim 8 wherein said immunoglobulin comprises heavy and light chains.

12. The transgenic mammal of claim 8 wherein said immunoglobulin comprises a single polypeptide chain.

13. The transgenic mammal of claim 8 wherein said immunoglobulin is of human origin.

14. An isolated purified DNA comprising in the 5' to 3' direction

a) 5' promoter sequences from the beta casein gene,

b) a unique Xho I restriction site, and

c) 3' untranslated sequences from the goat beta casein gene, wherein a) comprises nucleotides -6168 to -1 of the goat beta casein, wherein nucleotide 1 is the first nucleotide of the beta casein translation initation codon, b) comprises the sequence CGCGGATCCTCGAGGACC, and c) comprises the sequence

starting at the PpuMI site found at bp648 of the beta casein cDNA sequence, and continuing for 7.1 kb downstream, termininating in the sequence TAAGGTCCAGAGACCGAGACCCACTCACTAGGCAACTGGTCCGRCCAGCTGTTAAGTGA.

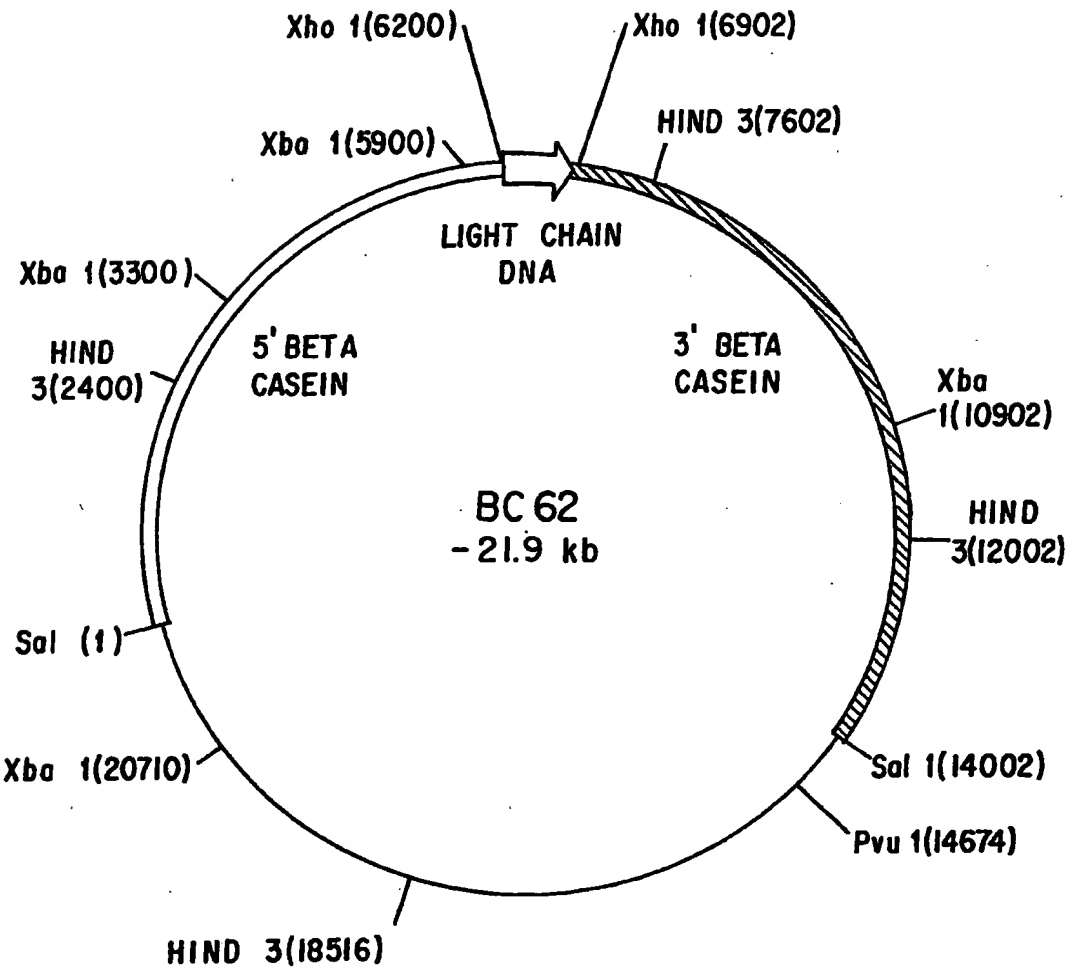
15. The DNA of claim 14 wherein an immunoglobulin cDNA is inserted into b), said DNA directing the mammary-gland-specific expression of said immunoglobulin in transgenic animals.

16. The DNA of claim 15 wherein said immunoglobulin comprises heavy and light chains.

17. The DNA of claim 15 wherein said immunoglobulin comprises a single polypeptide chain.

18. The DNA of claim 15 wherein said immunoglobulin is of human origin.

FIG. 1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Xho 1(6902)
HIND 3(7602)
Xba 1(5900)
LIGHT CHAIN DNA
Xba 1(3300)
HIND 3(2400)
5' BETA CASEIN
3' BETA CASEIN
Xba 1(10902)
BC 62
-21.9 kb
HIND 3(12002)
Sal (1)
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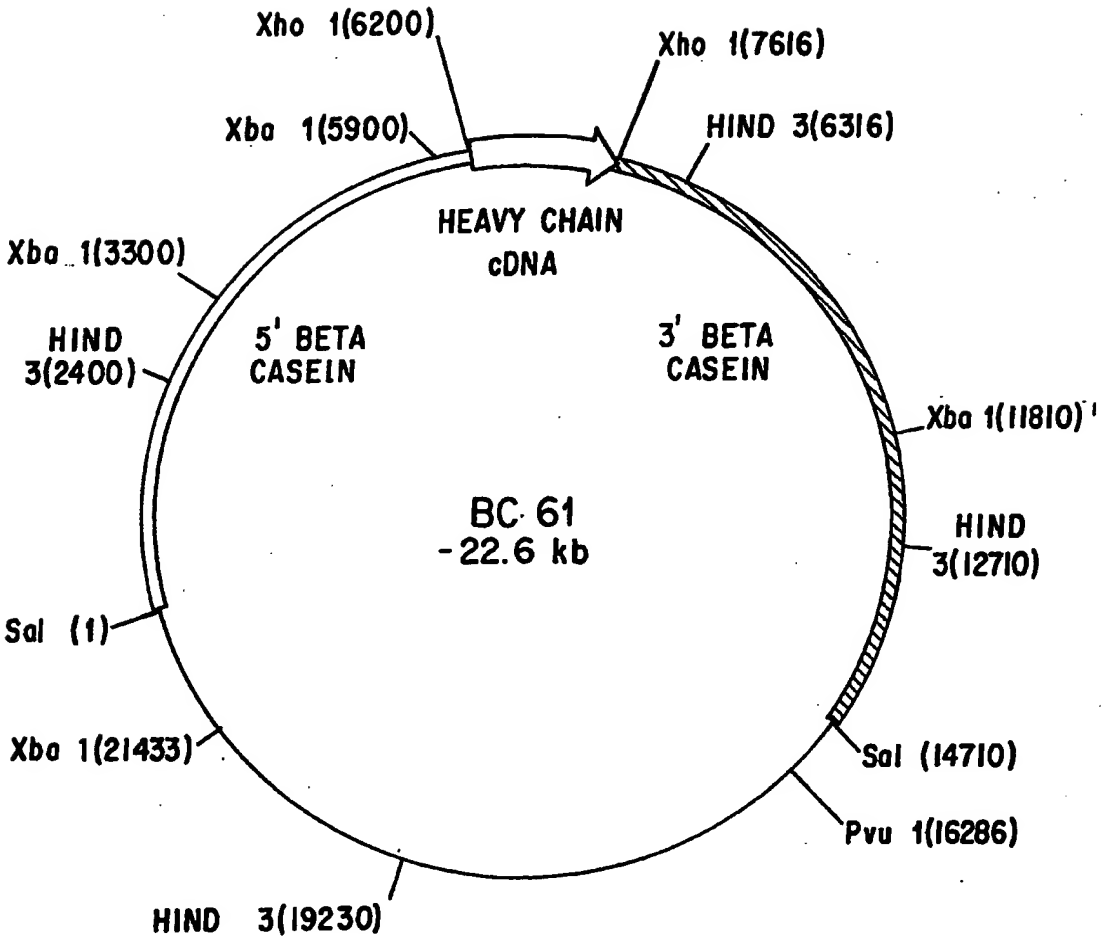
Pvu 1(14674)
HIND 3(18516)

FIG. 2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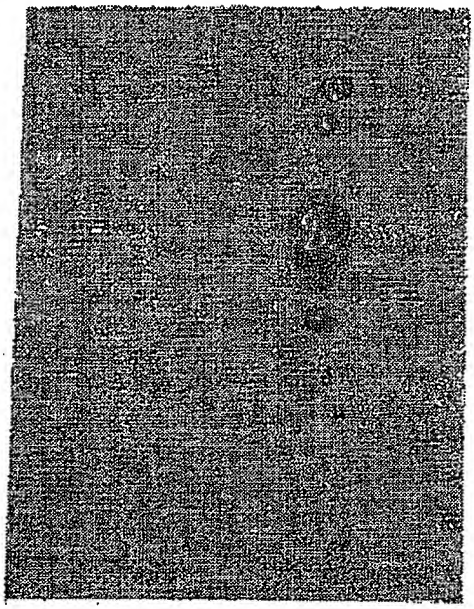
Xho 1(7616)
Xba 1(5900)
HIND 3(6316)
HEAVY CHAIN
cDNA
Xba 1(3300)
HIND
3(2400)
5' BETA
CASEIN
3' BETA
CASEIN
Xba 1(11810)
BC 61
-22.6 kb
HIND
3(12710)
Sal (1)
Xba 1(21433)
Sal (14710)
Pvu 1(16286)
HIND 3(19230)

FIG. 3



CD-1
1-15
1-23 D7
1-23 D9
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2-76 D7
2-82 D7
2-95 D7
2-92 D7
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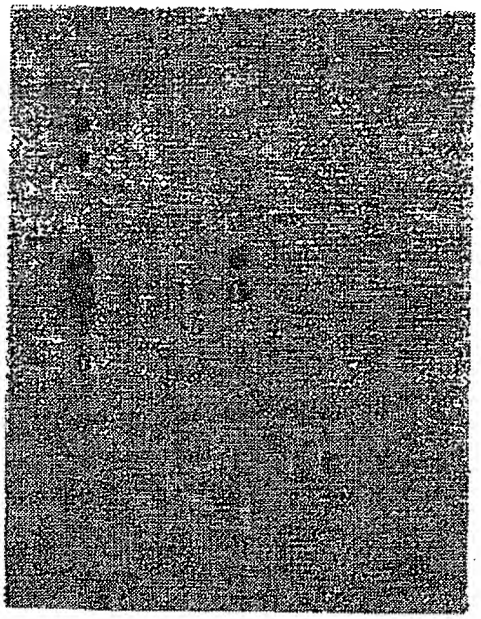
50 NG
100NG

FIG. 4



CD-1
1-15
1-23 D7
1-23 D9
1-24 D7
1-24 D9
2-76 D7
2-82D7
2-95D7
2-92 D7
X
50NG
100NG

INTERNATIONAL SEARCH REPORT

Ii. national application No.
PCT/US94/14795

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01K 67/00; C07H 21/00; C12N 15/00
US CL :435/172.3, 320.1; 536/23.53, 24.1; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 320.1; 536/23.53, 24.1; 800/2

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Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE, A, 40,00,939 (BREM) 18 July 1991, see the entire document.	1-13
P,Y	US, A, 5,322,775 (CLARK ET AL) 21 June 1994, see the entire document.	1-13
Y	Bio/Technology, Volume 9, issued September 1991, K.M. Ebert et al, "Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: Generation of transgenic goats and analysis of expression", pages 835-838, see the entire document.	1-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
16 MARCH 1995	29 MAR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRUCE CAMPELL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14795

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEBS Letters, Volume 284, No. 1, issued June 1991, M.G. Stinnakre et al, "The bovine α-lactalbumin promoter directs expression of ovine trophoblast interferon in the mammary gland of transgenic mice", pages 19-22, see the entire document.	1-13
Y	Gene, Volume 121, issued 1992, B. Roberts et al, "Cloning of the goat β-casein-encoding gene and expression in transgenic mice", pages 255-262, see the entire document.	1-18
Y	US, A, 4,873,316 (MEADE ET AL) 10 October 1989, see the entire document.	1-13
Y	FASEB Journal, Volume 7, No. 7, issued 30 May 1993, P. Ditullio et al, "High level expression of tissue plasminogen activator using the goat beta casein promoter", page A1223, abstract no. 993, see the abstract.	1-18
Y	Nature, Volume 355, issued 16 January 1992, M.A. Duchosal et al, "Immunization of hu-PBL-SCID mice and the rescue of human monoclonal Fab fragments through combinatorial libraries", pages 258-262, see the entire document.	1-18

INTERNATIONAL SEARCH REPORT

In...national application No.
PCT/US94/14795

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14795

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, CA, DERWENT, AGRICOLA, MEDLINE, EMBASE
search terms: transgenic animal, milk, antibody,casein, beta lactoglobulin, whey acidic protein, lactalbumin, promoter, goat beta casein gene

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-13, drawn to transgenic mammals and methods for production of immunoglobulins in milk.

Group II, claims 14-18, drawn to DNA constructs.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The product of Group II and the product of Group I are linked as intermediate and final product. The DNA constructs of Group II (intermediate) can be used to produce transgenic animals expressing proteins other than immunoglobulins (claim 14 is not limited to immunoglobulins). The constructs of Group II can also be used for other purposes, such as production of proteins in in vitro cell cultures. Furthermore, the broadest claims of Group I do not require the specific regulatory sequences recited in the claims of Group II. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.